DEVELOPMENTAL BIOLOGY **196,** 184–192 (1998)
ARTICLE NO. DB988866

Ectoderm Cell–ECM Interaction Is Essential for Sea Urchin Embryo Skeletogenesis

Francesca Zito,* Valentina Tesoro,* David R. McClay,† Eizo Nakano,‡ and Valeria Matranga*

**Istituto di Biologia dello Sviluppo del Consiglio Nazionale delle Ricerche, Via Ugo La Malfa 153, I-90146 Palermo, Italy; †Developmental, Cellular, and Molecular Biology Group, LSRC, Duke University, Durham, North Carolina 27708; and ‡Nagoya University, Chikusa, Nagoya 464, Japan*

Paracentrotus lividus **sea urchin nectin (*Pl*-nectin) is an extracellular matrix (ECM) protein of the sea urchin embryo on the apical surface of the ectoderm and has been shown to be an adhesive substrate for embryonic cells. A monoclonal antibody (McAb) to *Pl*-nectin was generated that inhibits the adhesion of blastula cells to *Pl*-nectin-coated substrates in an *in vitro* functional assay. To examine for possible *in vivo* functions of *Pl*-nectin, Fab fragments (Fabs) of *Pl*-nectin McAb were added to early blastulae. Ingression of primary mesenchyme cells was not affected by Fabs. As control embryos reached the pluteus stage, treated embryos showed a severe inhibition of skeletal elongation and patterning. When the Fabs were injected directly into the blastocoel, even at higher concentration than was applied externally, skeletogenesis was normal. Therefore, the effect of the antibody on spiculogenesis was indirect. The treatment was partially reversible as embryos eventually seemed to recover and elongate spicules, although with an incorrect patterning. Migration of pigment cells was also affected by the Fabs, since they did not disperse throughout the ectoderm but remained clustered in ectopic areas. In contrast, the development of endoderm structures was not affected. Our results indicate that in the sea urchin embryo the appropriate contact of ectodermal cells with outer ECM components is essential for the correct morphogenesis of inner mesodermal structures.** © 1998 Academic Press

Key Words: **sea urchin embryo; ECM protein; morphogenesis; skeletogenesis; pigment cells.**

INTRODUCTION

ECM proteins are known to play essential roles in morphogenetic movements that occur early in the development of most organisms. A direct correlation between the first appearance of ECM proteins and sites of embryonic cell movements has been demonstrated in many studies. In amphibian embryos, for example, fibronectin (FN) appears on the blastocoelic roof where mesodermal cells will later migrate (Boucaut *et al.*, 1990). Similarly, in avian embryos FN has been localized at the beginning of gastrulation in neural crest cell migration pathways (Thiery *et al.*, 1982; Duband and Thiery, 1982). One approach to analyzing the role of ECM in development and morphogenesis has been to investigate the consequences of the addition of antibodies specific to ECM molecules. Microinjection into the blastocoelic cavity of amphibian embryos of anti-FN antibodies causes blockage of gastrulation while it interferes with migration of neural crest cells in avian embryos (Boucaut *et al.*, 1984; Bronner-Fraser, 1986). Similarly, in the chick embryo perturbation of the normal migration pattern of primitive streak cells is caused by treatment with anti-laminin antibodies (Zagris and Chung, 1990).

Because of its relative simplicity the sea urchin embryo has been a useful system for studying the role of cell–cell and cell–ECM interactions during epithelial invagination, during cell rearrangements, and during mesenchymal patterning that occurs in early development. An extensive literature on many aspects of the biology of the sea urchin embryo is available (for reviews see Giudice, 1986; Hardin, 1996). In this embryo most of the ECM components described in vertebrate systems have been detected inside the blastocoel, in the basal lamina, or in the extraembryonic matrix. The restricted expression of some of these ECM molecules has been correlated to the time and site of mor-

0012-1606/98 $25.00

phogenetic and differentiating events, although in most cases that presence alone is not sufficient to assign a biological function. One approach used to analyze the role of cell–ECM interactions during morphogenesis involves function-blocking antibodies. Blastocoelic microinjection of antibodies to the ECM 1 epitope and ECM 18 resulted in perturbation of archenteron morphogenesis in one case (Ingersoll and Ettensohn, 1994) and PMC and endoderm cell organization in the other (Berg *et al.*, 1996). Other *in vivo* biological studies on ECM molecules that surround the embryo involved addition of antibodies to the culture medium outside the embryos. Burke *et al.* (1991) demonstrated that treatment of embryos with McAbs against apical lamina blocks the initial phase of gastrulation and produces partial exogastrulae. They did not observe any effect on secondary mesenchyme cell (SMC) movements nor on spicule formation. McAbs against hyalin inhibited both gastrulation and spiculogenesis (Adelson and Humphreys, 1988). In treated embryos pigment cells appeared at the scheduled time, and spicules were formed even if they failed to grow normally. The authors suggested that normal arm extension involves a feedback interaction between the spicule-forming mesodermal cells and the hyalin-binding ectodermal cells.

Recently we purified an ECM protein from unfertilized eggs of *Paracentrotus lividus* by affinity chromatography on a gelatin–Sepharose column (Matranga *et al.*, 1992). This protein, called *Pl*-nectin, is a 210-kDa homodimer consisting of two polypeptides with an equal mass of 105 kDa each, jointed covalently by S–S bridges. *Pl*-Nectin is stored in granules that are uniformly distributed throughout the unfertilized egg cytoplasm. It is released into the ECM surrounding the embryo after fertilization and, in later developmental stages, polarized on the apical surface of ectodermal and endodermal cells. *Pl*-Nectin supports the adhesion of blastula cells to the substrate, as shown by an *in vitro* adhesion assay.

In this report, we used McAbs to *Pl*-nectin for studying the *in vivo* biological function of the molecule. We find that inhibition of the interaction between ectodermal cells and the ECM causes indirectly an abnormal development of mesodermal structures. One interpretation of these observations is that a signal is normally transferred from ectoderm cells to the mesenchyme. When ectoderm cells are released from a normal interaction with their substrate, they no longer transmit the signal basally, and mesoderm, as a result, fails to differentiate its structures.

MATERIALS AND METHODS

Pl-Nectin Preparation

Pl-Nectin was isolated by affinity chromatography on gelatin–Sepharose as previously described (Matranga *et al.*, 1992). Unpublished results showed that *Pl*-nectin does not bind to Sepharose alone, but it specifically binds to gelatin. *Pl*-Nectin was stored at −20°C in 8 M urea/50 mM Tris, pH 7.5, until used for cell adhesion assays or it was dialyzed into 10 mM Tris, pH 7.5, just prior to use for SDS–PAGE.

Cell Surface Biotinylation and Protein Detection

Procedures for cell surface biotinylation were followed using an ECL protein biotinylation kit (Amersham) according to manufacturer's instructions. Briefly, eggs and embryos were washed twice in ice-cold Millipore filtered sea water (MFSW) and then incubated at 4°C in biotinylation reagent (Amersham) for 30 min. Eggs and embryos treated with biotin were washed twice with MFSW and lysed in a TBS lysis buffer (20 mM Tris, 0.5% Triton X-100, 150 mM NaCl, pH 7.5) containing a cocktail of protease inhibitors (2 μg/ml aprotinin, antipain, leupeptin, pepstatin A, benzamidine, and 2 mM phenylmethylsulfonyl fluoride) for 20 min on ice. For small-scale isolations of *Pl*-nectin, lysates were incubated with 200 μl of a 50% solution of gelatin–Sepharose (Pharmacia) for 2 h with constant rocking. The pellets were washed twice with 1% Triton X-100/50 mM Tris, pH 7.5, twice with 1 M NaCl/50 mM Tris, pH 7.5, and finally with 10 mM Tris, pH 7.5. Following resuspension in SDS–PAGE sample buffer and heating for 5 min at 100°C, gelatin-bounded proteins were separated by SDS–PAGE on a 6% gel according to Laemmli (1970). Following electrophoresis, proteins were transferred to nitrocellulose membranes according to Towbin *et al.* (1979) and incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Amersham) for 1 h. Biotinylated proteins were detected using chemiluminescence (Amersham's ECL detection kit) according to manufacturer's instructions.

Production and Characterization of Monoclonal Antibodies

Pl-Nectin antigen was excised from 6% polyacrylamide gels of affinity-purified protein, and the gel slices were Dounce-homogenized and injected intraperitoneally in Balb-c mice. Splenic cells from the mouse with the highest positive titer were fused to myeloma cells Ag8653 according to Galfre *et al.* (1977). Hybridoma cell supernatants were screened for anti-*Pl*-nectin reactivity by immuno-dot-blot, using purified *Pl*-nectin as antigen, and by indirect immunofluorescence, on sections of unfertilized eggs embedded in paraffin. Positive clones were subcloned by limiting dilution and rescreened on Western blots of purified *Pl*-nectin. In preliminary experiments we discovered that 3 of 15 different *Pl*-nectin-recognizing IgGs, namely clones IA12e9, VIE11h7, and VC5c8, affected normal development. We chose McAb IA12e9 for further investigation in order to be consistent with the biochemical data described. IgGs from hybridoma cell supernatants were purified by affinity chromatography on protein A–Sepharose CL-4B (Pharmacia) as described by Goding (1978) and concentrated by ultrafiltration (Amicon) to approximately 10 mg/ml. Fab fragments were produced by papain digestion of purified IgGs according to Harlow and Lane (1988).

Cell–Substrate Adhesion Assay

To measure the adhesion of cells to the substrate we utilized an assay originally developed by McClay and Fink (1982) with some modifications as described by Matranga *et al.* (1992). Purified *Pl*-nectin (10 μg/ml) was bound to microtiter plate wells for 3 h at room temperature. Blanking of the wells was performed by incuba-

tion with 10 mg/ml bovine serum albumin (BSA) overnight at 4°C. Incubation with several antibody concentrations was performed for 1 h at room temperature and then wells were washed three times with MFSW. *Paracentrotus lividus* embryos were metabolically labeled by culturing them with 0.6 μCi/ml of [^{3}H]lysine (Amersham) until the mesenchyme blastula stage was reached and dissociated into single cells by the technique fully described by Matranga *et al.* (1986). Radiolabeled cells, 1×10^5 per well, were settled onto *Pl*-nectin-coated wells for 1 h at 1*g*, and then the plates were inverted and spun off for 8 min at 100*g*. The percentage of cells bound was calculated relative to the radioactivity associated to 1×10^5 ^{3}H-labeled cells counted separately. Reported values are the means of four replicates for which the standard error is calculated. Values of controls made by plating cells onto BSA, giving typically about 10–20% of unspecific binding, were subtracted.

Perturbation Assay

To test the effects on morphogenesis of antibodies, varying amounts of purified Fab fragments were added to microtiter plate wells, containing early blastulae (8 h postfertilization at 16°C). Embryos, between 20 and 70 per well, were allowed to develop at 16°C and scored by optical inspection or photographic recording. To follow the development of single embryos, chamber slides were prepared by placing two pieces of double-stick Scotch adhesive tape along the short edges of a slide, pipetting embryos, and then placing a coverslide on the assemblage. In some cases embryos were monitored by microscopy and photographed on a Leitz microscope.

Immunofluorescence

Indirect immunofluorescence on sections embedded in paraffin was performed using McAb to *Pl*-nectin as previously described (Matranga *et al.,* 1992). Indirect immunofluorescence on whole-mount embryos was modified after Coffman and McClay (1990). Briefly, embryos treated or microinjected with Fabs to *Pl*-nectin were fixed for 2 min in ice-cold 0.1% formalin, permeabilized for 2 min in ice-cold MetOH, and then rinsed in MFSW. Embryos were incubated for 30 min in FITC-conjugated rabbit anti-mouse IgG (Sigma) diluted 1:100 in MFSW. After washing three times in MFSW, the embryos were observed under a Zeiss fluorescent microscope and photographed, using Ilford HP5 400 ISO film.

Microinjection

Sea urchin embryos were prepared for microinjection using a method modified by McMahon *et al.* (1985). Embryos at the early blastula stage were dejellied in MFSW, pH 4.5, and, after washing in normal MFSW, were electrostatically fixed to 60-mm Petri dish lids treated with 1% protamine sulfate, using a mouth pipet. Microinjection needles, from Eppendorf, were back-filled with a solution of IA12e9 Fabs, at a concentration of 10 μg/μl in MFSW. The blastocoel of immobilized embryos received approximately 100 pl of the antibody solution. The final concentration of the Fabs in the blastocoel after injection was 1.9 mg/ml, assuming the volume of the injected blastocoel is 523 pl. The apparatus for microinjection used was a Narishige IM-188. Following microinjection, embryos were collected with a mouth pipet, allowed to develop in microtiter plate wells at 16°C, and photographed as above.

RESULTS

Localization of Pl-Nectin in Eggs and Embryos

Previous work provided evidence that *Pl*-nectin is polarized to the apical surface of ectodermal and endodermal cells at late stages of embryo development (Matranga *et al.*, 1992). To verify that *Pl*-nectin is located extracellularly, a cell surface biotinylation procedure was utilized. Whole embryos were incubated with biotin at 4°C to prevent internalization and consequently labeling of cytoplasmic proteins. Embryo lysates were incubated with gelatin–Sepharose, bound proteins were run on SDS–PAGE and transferred to nitrocellulose membranes, and biotinylated proteins were detected by HRP–streptavidin binding and enhanced chemiluminescence. Unfertilized eggs, where *Pl*-nectin is found in cytoplasmic granules, were used as controls. Results of this experiment, together with indirect immunofluorescence showing the localization of the molecule in eggs and late gastrula embryos, are shown in Fig. 1. Clearly *Pl*-nectin from gastrula embryos is biotinylated (Fig. 1D), demonstrating that the protein is located extracellularly at this developmental stage (Fig. 1B). On the contrary, *Pl*-nectin from eggs, although affinity-purified by gelatin–Sepharose (Fig. 1C), is not labeled (Fig. 1D) and is therefore entirely contained within the unfertilized egg (Fig. 1A).

McAbs to Pl-Nectin Inhibit Cell–Substrate Adhesion

We have shown previously that *Pl*-nectin could serve as an adhesive substrate for sea urchin cells (Matranga *et al.*, 1992). To characterize this interaction *in vivo* we developed function blocking antibodies to *Pl*-nectin. The McAbs used in this study were prepared using as immunogen the band of *Pl*-nectin cut from SDS–PAGE gels. We selected and subcloned those hybridomas whose supernatants were positive for inhibition of cell adhesion by both an *in vitro* assay and Western blot (Zito, 1995). The hybridoma supernatant IA12e9, which best inhibited cell adhesion to *Pl*-nectin-coated substrates, was selected and IgGs were affinity purified. The effects of IgGs in preventing the adhesion of mesenchyme blastula cells to *Pl*-nectin-coated substrates were tested by the *in vitro* adhesion assay. The concentration of affinity-purified *Pl*-nectin used to coat the microtiter wells was 10 μg/ml. Incubation with IgGs, at concentrations of 7, 14, and 28 μg/ml, was performed prior to the addition of cells. As shown in Fig. 2 increasing amounts of IA12e9 IgG produced a decrease in cell binding to the substrate in a dose-dependent manner. We calculated a 45% inhibition of cell adhesion when using the minimal IgG concentration and an inhibition as high as 70% with the maximal dose used. Unrelated IgG did not interfere with the binding of cells to *Pl*-nectin.

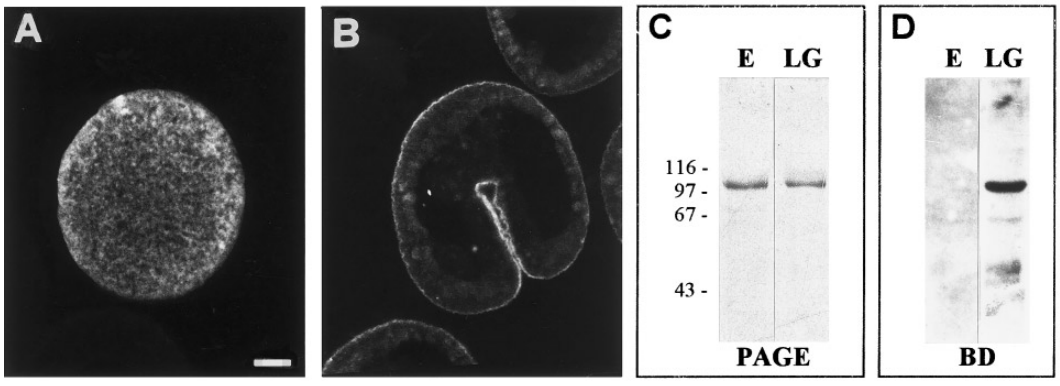

FIG. 1. *Pl*-Nectin is localized extracellularly in embryos at the late gastrula stage. Polyclonal antibodies to *Pl*-nectin were used in indirect immunofluorescence on sections from *Paracentrotus lividus* eggs (A) and late gastrula embryos (B). The antigen is found in cytoplasmic granules in eggs and on the outside of the embryo at gastrula stage. (C) Lysates from eggs (E) or late gastrula embryos (LG), previously incubated with biotin, were adsorbed to gelatin-Sepharose and bound proteins run on SDS–PAGE. Only one band at 105 kDa is found which correspond to *Pl*-nectin. (D) Biotin detection (BD) by HRP-conjugated streptavidin of gelatin-Sepharose bound proteins transferred to nitrocellulose. A 105-kDa protein is found biotinylated only in late gastrula lysates.

Perturbation of Embryonic Development by McAbs to Pl-Nectin

It has been suggested that molecules in the ECM surrounding the embryo are substrates for morphogenetic movements at gastrulation. We asked therefore whether McAbs specific to *Pl*-nectin would affect normal development. Early blastula embryos (8 h postfertilization) were cultured in the presence of Fabs purified from IgGs to *Pl*-nectin. The development of treated embryos was monitored at different time intervals and compared to embryos cultured in the presence of unrelated Fabs.

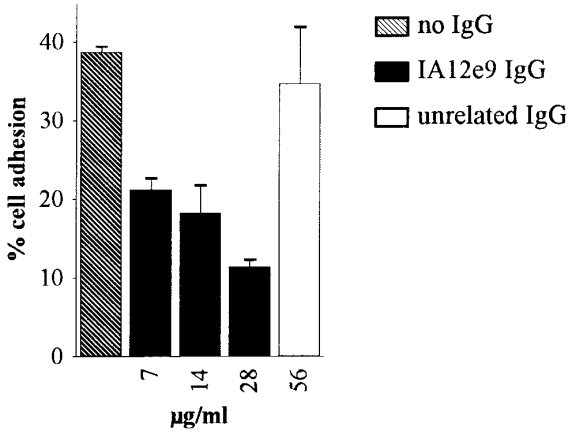

FIG. 2. McAb to *Pl*-nectin inhibit adhesion of dissociated cells on *Pl*-nectin-coated substrates. Radiolabeled cells dissociated from mesenchyme blastula embryos were used at a dilution of 10^5 cells per well. Each substrate well was coated with *Pl*-nectin at 10 µg/ml. IgG were purified from tissue culture supernatants of the hybridoma clone IA12e9 and added to the wells at the concentrations shown. Rabbit anti-mouse IgGs were used as unrelated IgG. The data shown represent the averages of four replicates ±SE, from a typical experiment conducted three times.

Between 4 and 10 h of treatment with McAb IA12e9, embryos appeared indistinguishable from controls: PMCs ingressed into the blastocoelic cavity on time and with the usual pattern, and the archenteron began to invaginate at the expected time (not shown). The initiation of skeletogenesis was not affected since treated embryos showed triradiate spicule rudiments as did the controls (not shown).

A severe inhibition of skeletal patterning was observed after 42 h of treatment. While control embryos were well-developed plutei (Fig. 3A), treated embryos showed characteristic skeletal abnormalities: a failure either in correct patterning (Fig. 3B) or in branching of skeletal rods (Fig. 3C). As a consequence, treated embryos had poorly developed arms and maintained the spherical shape characteristic of the blastula and gastrula stages (Figs. 3B and 3C). Thus, exposure of the apical side of ectoderm cells to the antibody had an effect on skeletogenesis, even though skeletogenesis occurs in the bastocoel to the basal side of these ectodermal cells. The morphology of ectodermal cells changed in ways that appeared similar to control embryos. The aboral ectoderm changed from cuboidal to squamous epithelium as in controls, while the oral ectoderm cells at the animal cap elongated to form the characteristic animal cap thickening, again as in controls. Similarly, endoderm derivatives were not inhibited in their differentiation by anti-*Pl*-nectin antibody, as the three parts of the digestive apparatus were properly organized (Fig. 3B). The effects observed are quantified in Table 1. The embryos were scored on an arbitrary scale, where the index of skeleton deficiency (ISD) has been expressed in units from 1 to 4. Type 1 is normal embryo (see

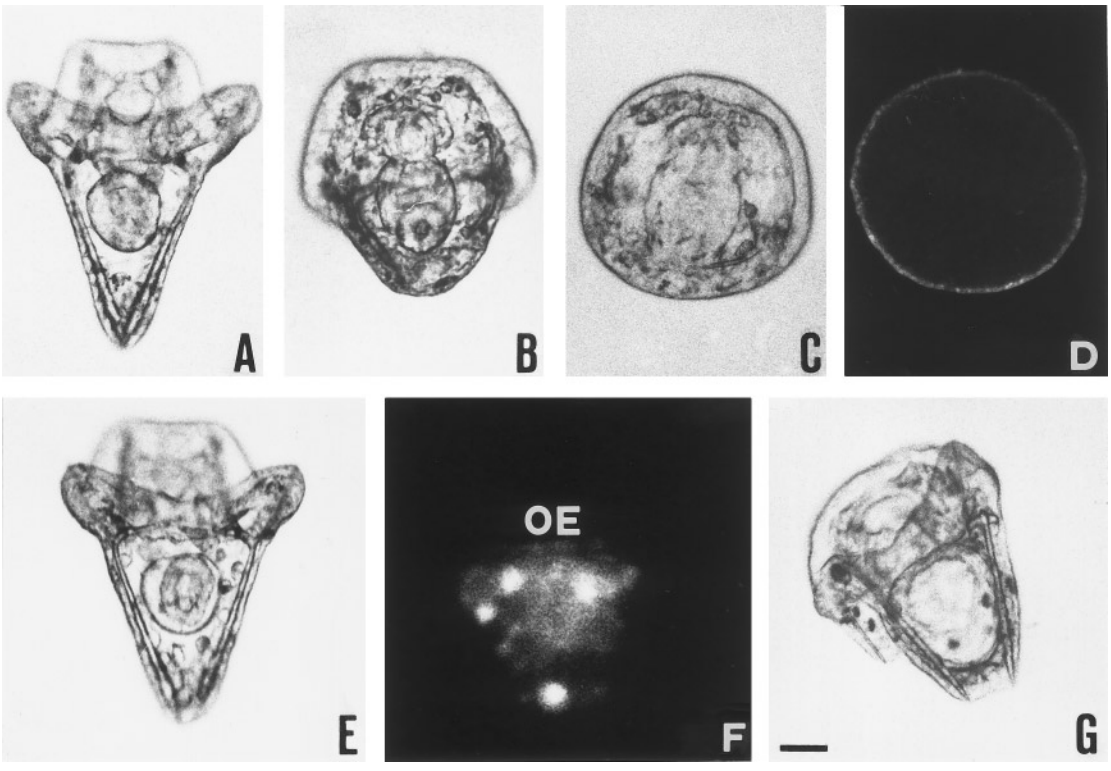

FIG. 3. Exposure of embryos to anti *Pl*-nectin McAb causes deficiencies in skeletogenesis and arm elongation. Antibodies were added to the culture (A–D, G) or injected into the blastocoel (E, F) always at the early blastula stage (8 h postfertilization). Control embryos cultured in the presence of unrelated Fabs for 42 h are normal pluteus larvae (A), while embryos cultured in the presence of IA12e9 Fabs show no arms and undersized skeletal rods (B, C). Depending on the severity of abnormalities, embryos have been classified as type 3 or type 4, respectively, on an arbitrary scale where the index of skeleton deficiencies (ISD) has been evaluated on a scale from 1 to 4. Type 1 is normal embryo (A) and type 4 is extreme skeleton deficiency (C). Embryos of the same batch of C were fixed and processed with FITC-conjugated secondary antibody, showing staining only on the extraembryonic ECM (D). Embryos injected with 1 ng of IA12e9 Fabs at the early blastula stage, observed 42 h after injection, develop normally and form fully elongated arms and a normal skeleton (E). Embryos from the same batch as in E, treated for immunofluorescence as above, show a diffuse immunofluorescence inside the embryo (F). OE, oral ectoderm. A partial recovery of skeletogenesis is obtained after 64 h in Fabs without refreshing (G). Bar = 20 μm.

Fig. 3A) and type 4 is maximum skeleton deficiency (see Fig. 3C). This scale is conceptually similar to the index of axis deficiency (IAD), defined by Scharf and Gerhart (1980), in the Xenopus embryo. The effects of different amounts of anti-*Pl*-nectin McAb were also tested. Increasing concentrations of Fabs produced more severe effects (Table 1).

We next asked whether there would be an inhibition of spicule elongation and patterning if antibodies were applied directly to the PMCs. One nanogram of Fabs in a volume of 100 pl was injected into the blastocoelic cavity of early blastula embryos and development was followed. The final concentration of Fabs in the blastocoel was calculated to be 1.9 mg/ml, roughly the same concentration used outside the embryo. Injected embryos (Fig. 3E) were indistinguishable from controls (Fig. 3A) and reached the pluteus stage at the same time as controls.

The inhibition of skeletogenesis was partially reversed with continuing treatment. After 64 h of treatment some perturbed embryos eventually recovered and showed a pluteus-like morphology, i.e., the spicule rudiments continued extending though their patterning never became completely normal (Fig. 3G).

To ask if antibodies were actually binding to the apical surface of ectoderm cells, 42-h externally treated embryos were fixed and stained with FITC-conjugated anti-mouse antibodies. A fluorescence signal was detected on the apical surface of the embryos, and no signal was found inside the blastocoel (Fig. 3D). When embryos that had been injected with the Fab antibody were fixed and stained with secondary antibody, a signal was found diffusely within the blastocoel (Fig. 3F).

Since treatment of embryos with McAbs against *Pl*-nectin resulted in some morphogenetic abnormalities, we asked whether tissue differentiation was also affected. Embryos

TABLE 1
Effect of McAb Anti-*Pl*-Nectin on Skeleton Elongation and Patterning

Trial No.	Time (h)[b]	Fab (μg/μl)	Total embryos	Morphology[a]			
				Type 1	Type 2	Type 3	Type 4
1	42	2.0	15	0	0	6	9
2	42	2.0	29	0	0	16	13
3	42	2.0	16	1	0	7	8
						Type 3 + 4	
4a	42	1.5	72	63	0	9	
4b	42	2.0	44	20	0	24	
4c	42	2.5	30	12	0	18	
5a	64	1.5	68	54	11	3	
5b	64	2.5	46	33	7	6	

[a] Morphologies were estimated on an arbitrary scale from 1 to 4 as follows: type 1, pluteus (no perturbation of development, see Fig. 3A); type 2, pluteus-like shape with abnormal skeleton patterning (see Fig. 3G); type 3, trapezoidal shape with about half-sized skeletal rods (see Fig. 3B); type 4, spherical shape with unbranched spicules (maximum of skeleton deficiency, see Fig. 3C).

[b] Period of culture of embryos in the presence of McAb, which was always added at the early blastula stage (8 h postfertilization).

treated for 42 h with anti-*Pl*-nectin antibody were fixed and stained with McAbs markers to the ectoderm, mesoderm, and endoderm territories. We used the following McAbs: Ecto V which detects an antigen that becomes restricted to the oral ectoderm and foregut (Coffman and McClay, 1990); ID5, a highly specific PMC marker (Hardin *et al.*, 1992); Endo 1, a midgut and hindgut marker (Wessel and McClay, 1985); and UH2-95 that is specific for the ciliary band (Adelson, 1985), a structure arising late in development at the border separating the oral and aboral ectoderm (Cameron *et al.*, 1993). In all cases the antigens appeared in control and treated embryos at the appropriate time and in the correct position (not shown). Thus, while abnormal skeletogenesis occurs in antibody-treated embryos, the germ layer and territory-specific markers appear in appropriate patterns.

In some experiments we noticed that embryos treated for 42 h with McAb to *Pl*-nectin had a peculiar distribution of pigment cells (Fig. 4). These cells, which originate from the differentiation of SMCs, did not migrate and distribute evenly throughout ectoderm cells as they usually do (Gibson and Burke, 1985). On the contrary, we found that in some of the treated embryos, pigment cells clustered at the animal pole and at the sides of the vegetal plate (Fig. 4B). In few other cases pigment cells were found arranged in a subequatorial ring with some cells dispersed in the vegetal ectoderm (Figs. 4C and 4D).

DISCUSSION

In a previous paper we showed that *Pl*-nectin has an *in vitro* biological activity as an adhesion substrate molecule. Further, the pattern of expression of *Pl*-nectin suggested it plays a functional role during sea urchin embryo morphogenesis. The experiments described in this paper begin to address details on both the molecule itself and its functional role in the embryo.

McAbs to Pl-Nectin Indirectly Affect PMC Morphogenesis

To understand the *in vivo* biological role played by *Pl*-nectin during morphogenesis, we tried to interfere with its activity by using McAbs to *Pl*-nectin on *P. lividus* embryos. The effect of the antibody was not obvious until the midgastrula stage. PMCs ingressed normally and on schedule, indicating that the presence of the McAb did not affect their differentiation. PMCs lose affinity to a number of substrates as they ingress (Fink and McClay, 1985; Burdsal *et al.*, 1991), so the inhibition of PMC adhesion with an antibody might not be expected to block ingression in any event. However, embryos treated with McAb IA12e9 failed to elongate spicule rods. The antibodies did not block spiculogenesis by binding directly to PMCs since antibody placed in the blastocoel had no effect. *Pl*-Nectin is located in the extraembryonic ECM yet the inhibitory effect observed was an altered spiculogenesis. Thus, we conclude the effect to be indirect. An inhibition of the ectoderm–*Pl*-nectin interaction somehow prevents the ectoderm from providing the correct patterning cues for spicule morphogenesis.

A general toxic effect of McAb IA12e9 on development is unlikely for several reasons. First, the perturbation was specific to the IA12e9 *Pl*-nectin-recognizing antibody. Other McAbs to *Pl*-nectin, possibly directed toward differ-

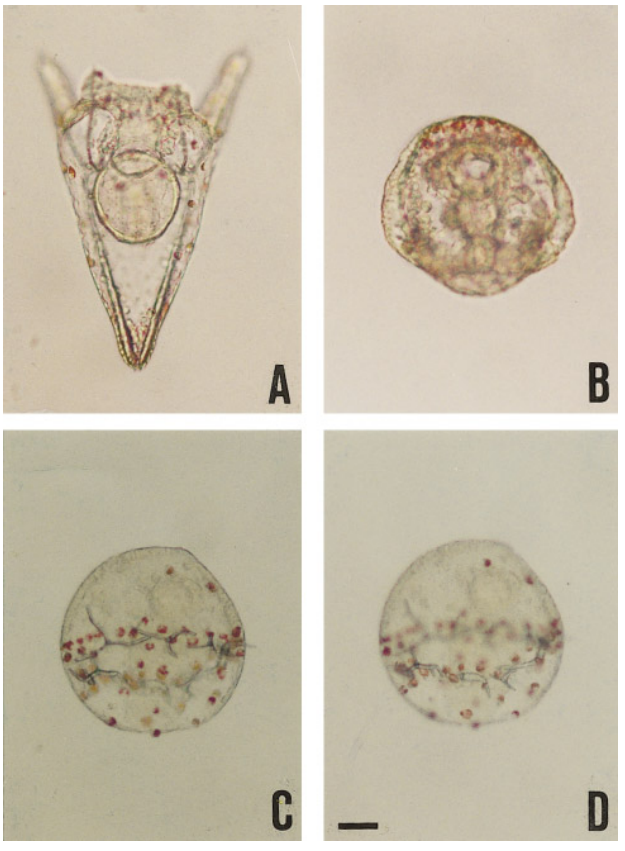

FIG. 4. Ectopic distribution of pigment cells in embryos treated with McAb to *Pl*-nectin. Control embryo, cultured in the presence of unrelated Fabs for 42 h, shows pigment cells dispersed in the ectoderm (A). In embryos cultured in the presence of IA12e9 Fabs for 42 h (B–D), pigment cells cluster at the animal pole and at the sides of the vegetal plate (B) or in a subequatorial ring (C, D). Pictures of the same embryo taken at two different focal planes are provided (C, D). Bar = 20 μm.

ent epitopes of the molecule, at the same or at higher concentrations, had no effect on morphogenesis. Similarly, unrelated Fabs used at the same or at higher concentrations had no effect. Second, the perturbation effects observed were directed mainly to mesodermal structures (Figs. 3 and 4), while a generic toxic behavior might have been expected to randomly delay or inhibit the development of all tissues, particularly those exposed to the medium, such as the ectoderm. Third, the effects observed were later at least partially reversed (Fig. 3). This recovery could be the result of the Fab–*Pl*-nectin complex internalization as was the case of anti-ECM18 antibody which was detected within the cells after prolonged embryo culture (Berg *et al.*, 1996). Alternatively, new synthesis of *Pl*-nectin might overcome the inhibiting effect of the antibody. ECM molecules have been shown to be synthesized during all developmental stages (Coffman and McClay, 1990; Nakano *et al.*, 1990; Furhman *et al.*, 1992; Berg *et al.*, 1996). A trivial explanation would be denaturation of antibodies in high ionic strength, like sea water, for prolonged periods of time. Fourth, the effects of McAbs were dose-dependent in that increasing concentrations of Fabs resulted in an increase in severity of perturbation (Table 1).

McAbs to Pl-Nectin Prevent Pigment Cell Migration

During gastrulation, pigment cell precursors are released from the vegetal plate or from the tip of the archenteron, migrating first to the vegetal ectoderm and subsequently dispersing throughout the ectoderm, where they develop pigment granules (Gibson and Burke, 1985). In perturbed embryos the pattern of migration of pigment cells is altered, as they remain clustered in ectopic areas (Fig. 4). This finding is difficult to explain since very few data in the literature describe how pigment cells migrate and disperse throughout the ectoderm. In vertebrate systems several factors have been hypothesized to control the timing and pattern of pigment cell migration (for a review, see Erickson, 1993). Among those, the roles played by the ECM environment (Erickson and Goins, 1995), growth factors (Horikawa *et al.*, 1995), and integrins (Vink *et al.*, 1994) have been proposed. At this stage we cannot exclude any of these hypotheses to account for the abnormal migration of pigment cells throughout the ectoderm in the sea urchin embryo. It is reasonable to conclude however that pigment cells, like their PMC mesenchymal counterparts, require patterning cues from the ectoderm for correct distribution in the embryo. The anti-*Pl*-nectin-inhibited ectoderm may lose the capacity to appropriately direct pigment cell migration.

How Does Pl-Nectin Affect Skeletogenesis?

The most severe effect observed in this study was an inhibition of skeleton patterning and consequently of arm elongation, whereas no interference with the first steps of triradiate spicule deposition and elongation was observed. Two sorts of studies bear on the mechanism of spiculogenesis. First, spiculogenesis occurs semiautonomously *in vitro,* requiring only the presence of horse serum (Okazaki, 1975; McClay and Fink, 1982). An assumption has been that the horse serum might provide growth factors that are appropriate for spiculogenesis, though conclusive evidence of that idea has yet to be published. Second, while spicules grow *in vitro,* their correct patterning appears to require growth *in vivo.* It has been shown that the ectoderm provides both spatial and temporal information necessary for correct skeleton patterning (Ettensohn and McClay, 1986; McClay *et al.*, 1992; Hardin *et al.*, 1992; Ettensohn and Malinda, 1993; Armstrong *et al.*, 1993; Armstrong and McClay, 1994). Thus, the ectoderm is likely to provide two sorts of signals for PMCs. Unless PMCs *in vivo* provide

their own growth support factors, the ectoderm must supply those factors *in vivo* and the ectoderm also provides spatial patterning cues for spicule morphogenesis. We propose that ectodermal cells require contact with *Pl*-nectin-containing ECM on their apical side in order to be competent to provide patterning signals to PMCs at their basal side.

ACKNOWLEDGMENTS

Part of this work has been the subject of a Ph.D. thesis by F.Z. at the Department of Cellular and Developmental Biology, University of Palermo. V.T. was supported by a CNR fellowship. We are grateful to Dr. S. Sciarrino for continuous encouragement and help during the preparation of the manuscript. This work has been partially supported by CNR Trilateral Project Italy–USA–Japan to V.M., D.M., and E.N.

REFERENCES

Adelson, D. (1985). Monoclonal antibodies to developmentally regulated proteins in the sea urchin embryo. Ph.D. Dissertation, University of Hawaii.

Adelson, D. R., and Humphreys, T. (1988). Sea urchin morphogenesis and cell–hyalin adhesion are perturbed by monoclonal antibody specific for hyalin. *Development* **104,** 391–402.

Armstrong, N., Hardin, J., and McClay, D. R. (1993). Cell–cell interactions regulate skeleton formation in the sea urchin embryo. *Development* **119,** 833–840.

Armstrong, N., and McClay, D. R. (1994). Skeletal pattern is specified autonomously by the primary mesenchyme cells in sea urchin embryos. *Dev. Biol.* **162,** 329–338.

Berg, L. K., Chen, S. W., and Wessel, G. M. (1996). An extracellular matrix molecule that is selectively expressed during development is important for gastrulation in the sea urchin embryo. *Development* **122,** 703–713.

Boucaut, J. C., Darribere, T., Boulekbache, H., and Thiery, J. P. (1984). Prevention of gastrulation but not neurulation by antibodies to fibronectin in amphibian embryos. *Nature* **307,** 364–367.

Boucaut, J. C., Johnson, K. E., Darribere, T., Shi, D. L., Riou, J. F., Boulekbache, H., and Delarue, M. (1990). Fibronectin-rich extracellular material controls cell migration during amphibian gastrulation. *Int. J. Dev. Biol.* **34,** 139–147.

Bronner-Fraser, M. (1986). An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development *in vivo*. *Dev. Biol.* **117,** 528–536.

Burdsal, C. A., Alliegro, M. C., and McClay, D. R. (1991). Tissue-specific, temporal changes in cell adhesion to echinonectin in the sea urchin embryo. *Dev. Biol.* **144,** 327–334.

Burke, R. D., Myers, R. L., Sexton, T. L., and Jackson, C. (1991). Cell movements during the initial phase of gastrulation in the sea urchin embryo. *Dev. Biol.* **146,** 542–557.

Cameron, R. A., Britten, R. J., and Davidson, E. H. (1993). The embryonic ciliated band of the sea urchin, *Strongylocentrotus purpuratus*, derives from both oral and aboral ectoderm. *Dev. Biol.* **160,** 369–376.

Coffman, J. A., and McClay, D. R. (1990). A hyaline layer protein that becomes localized to the oral ectoderm and foregut of the sea urchin embryos. *Dev. Biol.* **140,** 93–104.

Duband, J. P., and Thiery, J. P. (1982). Appearance of fibronectin during chick embryo gastrulation. *Dev. Biol.* **94,** 337–350.

Erickson, C. A. (1993). From the crest to the periphery: Control of pigment cell migration and lineage segregation. *Pigment Cell Res.* **6,** 336–347.

Erickson, C. A., and Goins, T. L. (1995). Avian neural crest cells can migrate in the dorsolateral path only if they are specified as melanocytes. *Development* **121,** 915–924.

Ettensohn, C. A., and McClay, D. R. (1986). The regulation of primary mesenchyme cell migration in the sea urchin embryo: Transplantations of cells and latex beads. *Dev. Biol.* **117,** 380–391.

Ettensohn, C. A., and Malinda, K. M. (1993). Size regulation and morphogenesis: A cellular analysis of skeletogenesis in the sea urchin embryo. *Development* **119,** 155–167.

Fink, R. D., and McClay, D. R. (1985). Three cell recognition changes accompany the ingression of sea urchin primary mesenchyme cells. *Dev. Biol.* **107,** 66–74.

Furhman, M. H., Suhan, J. P., and Ettensohn, C. A. (1992). Developmental expression of echinonectin, an endogenous lectin of the sea urchin embryo. *Dev. Growth Differ.* **34,** 137–150.

Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W., and Howard, J. C. (1977). Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature* **266,** 550–552.

Gibson, A. W., and Burke, R. D. (1985). The origin of pigment cells in embryos of the sea urchin *Strongylocentrotus purpuratus*. *Dev. Biol.* **107,** 414–419.

Giudice, G. (1986). ''The Sea Urchin Embryo.'' Springer-Verlag, Berlin, Heidelberg.

Goding, J. W. (1978). Use of staphylococcal protein A as an immunological reagent. *J. Immunol. Methods* **20,** 241–253.

Hardin, J., Coffman, J. A., Black, S. D., and McClay, D. R. (1992). Commitment along the dorsoventral axis of the sea urchin embryo is altered in response to $NiCl_2$. *Development* **116,** 671–685.

Hardin, J. (1996). The cellular basis of sea urchin gastrulation. *Curr. Top. Dev. Biol.* **33,** 159–262.

Harlow, E., and Lane, D. (1988). ''Antibodies: A Laboratory Manual.'' Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Horikawa, T., Norris, D. A., Yohn, J. J., Zekman, T., Travers, J. B., and Morelli, J. G. (1995). Melanocyte mitogens induce both melanocyte chemokinesis and chemotaxis. *J. Invest. Dermatol.* **104,** 256–259.

Ingersoll, E. P., and Ettensohn, C. A. (1994). An N-linked carbohydrate-containing extracellular matrix determinant plays a key role in sea urchin gastrulation. *Dev. Biol.* **163,** 351–366.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Matranga, V., Kuwasaki, B., and Noll, H. (1986). Functional characterisation of toposomes from sea urchin blastula embryos by a morphogenetic cell aggregation assay. *EMBO J.* **5,** 3125–3132.

Matranga, V., Di Ferro, D., Zito, F., Cervello, M., and Nakano, E. (1992). A new extracellular matrix protein of the sea urchin embryo with properties of a substrate adhesion molecule. *Roux's Arch. Dev. Biol.* **201,** 173–178.

McClay, D. R., and Fink, R. D. (1982). Sea urchin hyalin: Appearance and function in development. *Dev. Biol.* **92,** 285–293.

McClay, D. R., Armstrong, N. A., and Hardin, J. (1992). Pattern formation during gastrulation in the sea urchin embryo. *Development Suppl.*, 33–41.

McMahon, A. P., Flytzanis, C. N., Hough-Evans, B. R., Katula, K. S.,

Britten, R. J., and Davidson, E. H. (1985). Introduction of cloned DNA into sea urchin egg cytoplasm: Replication and persistence during embryogenesis. *Dev. Biol.* **108,** 420–430.

Nakano, E., Iwata, M., and Matranga, V. (1990). Collagen-binding proteins in sea urchin eggs and embryos. *In* ''Mechanisms of Fertilisation, NATO ASI Series'' (B. Dale, Ed.), Vol. H45, pp. 645–652. Springer-Verlag, Berlin.

Okazaki, K. (1975). Spicule formation by isolated micromeres of the sea urchin embryo. *Am. Zool.* **15,** 567–581.

Scharf, S., and Gerhart, J. (1980). Determination of the dorso-ventral axis in eggs *of Xenopus laevis:* Complete rescue of uv-impaired eggs by oblique orientation before first cleavage. *Dev. Biol.* **79,** 181–198.

Thiery, J. P., Duband, J. L., and Delouvee, A. (1982). Pathways and mechanism of trunk avian neural crest cell migration and localisation. *Dev. Biol.* **93,** 324–343.

Towbin, T., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some application. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.

Vink, J., Dekker, S. K., Van-Leeuwen, R. L., Vermeer, B. J., Bruijn, J. A., and Byers, H. R. (1994). Role of beta 1 integrins in cell spreading and migration of human nevomelanocytes and dysplastic nevi cells on collagen type IV and laminin. *Pigment Cell Res.* **7,** 339–347.

Wessel, G. M., and McClay, D. R. (1985). Sequential expression of germ-layer specific molecules in the sea urchin embryo. *Dev. Biol.* **111,** 451–463.

Zagris, N., and Chung, A. E. (1990). Distribution and functional role of laminin during induction of the embryonic axis in the chick embryo. *Differentiation* **43,** 81–86.

Zito, F. (1995). Molecole della matrice extracellulare nell'embrione di riccio di mare. Ph.D. Dissertation, Università di Palermo.

Received for publication December 8, 1997
Accepted January 15, 1998